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14. ABSTRACT

The prostate cancer (PCa) metastasis processes are facilitated by proteolytic cascade involving cathepsin K (CatK). CatK expression level is higher in PCa bone metastatic sites than primary tumor or normal prostate tissues. The role of CatK in PCa skeletal metastasis, however, is not known. We first confirmed the expression of CatK in LNCaP, C4-2B, and PC3 PCa cell lines. Then, we observed the inhibitory effect of CatK inhibitor on the PCa cell invasion suggesting the role of CaK in PCa tumor invasion. Finally, we injected C4-2B cells into the tibiae of SCID mice and then the animals received either vehicle or Cat K inhibitor for 8 weeks either at the time of tumor cells injection (tumor establishment model) or 4 weeks after tumor cells were injected (tumor progression model). In the tumor establishment model, CatK inhibitor significantly prevented the establishment of mixed osteolytic/osteoblastic tibial tumors as were observed in vehicle-treated animals. In the progression model, CatK inhibitor diminished tumor-induced bone lesions. We conclude that CatK inhibitor is an effective drug to prevent the establishment and diminish progression of PCa growth in bone. The overall goal of this project is to identify a clinically relevant strategy to inhibit PCa skeletal metastasis.

15. SUBJECT TERMS
Cathepsin K, prostate cancer, bone metastasis, animal model

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Introduction

The **subject of this study** is: "Inhibition of prostate cancer (PCa) skeletal metastases by targeting cathepsin K". PCa is the most frequently diagnosed cancer in men and the second leading cause of cancer death among men in the United States. New treatments are urgently needed for patients known to have bone metastases and those who are at high risk for having bone metastases in PCa patients. Cathepsin K (CatK) is a cystine protease enzyme that can degrade collagen I, the organic matrix of the bone. CatK was found in breast cancer cells that are capable of bone resorption and CatK message RNA was also detected in CaP cell lines and in primary CaP and metastases. Importantly, the CatK expression in bone metastases was significantly higher than in primary CaP, while CatK in normal prostate tissues were negative. These findings suggest that CatK may play a critical role in tumor bone metastasis. The **purpose of this project** is to identify a clinically relevant strategy to inhibit prostate cancer skeletal metastasis. The **specific scopes** of this research are: 1) To determine the effects of CatK inhibitor and Zometa (ZA) alone or in combination on the establishment of CaP growth in bone, and determine if these effects are synergistic. 2) To determine the effects of CatK inhibitor and ZA alone or in combination on the progression of CaP growth in bone, and determine if these effects are synergistic.

Body

We have made great progresses on the tasks that involve a lot of animal work. Our originally plans are: **Project Task 1.** To determine the effects of CatK inhibitor and ZA alone or in combination on the establishment of PCa growth in bone, and determine if these effects are synergistic (Months 1-18); **Project Task 2.** To determine the effects of CatK inhibitor and ZA alone or in combination on the progression of PCa growth in bone, and determine if these effects are synergistic (Months 18-36). Since we received the funding, we started to work on both tasks. We first confirmed the expression of CatK in LNCaP, C4-2B, and PC3 PCa cell lines (Figure 1).

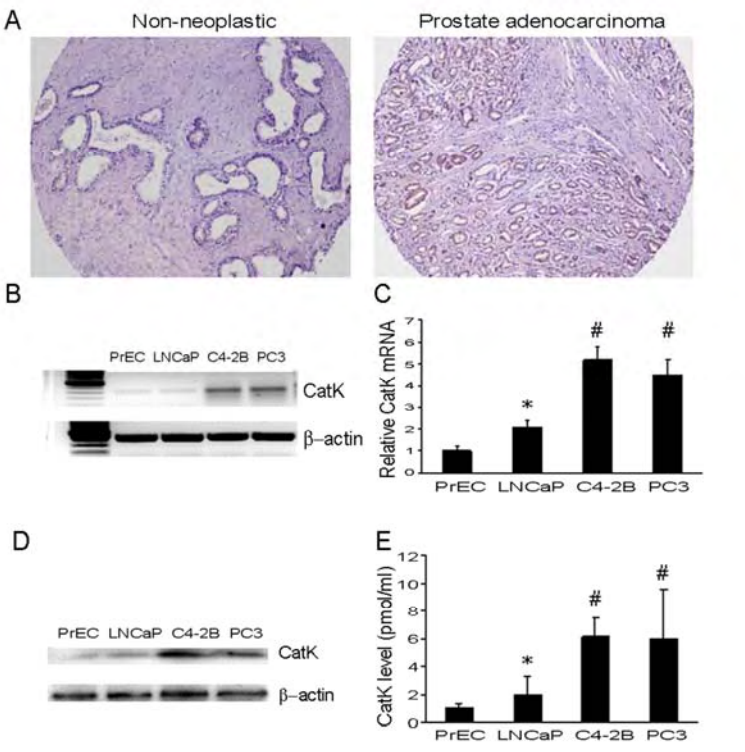

Figure 1. CatK mRNA and protein expression in PCa tissues and cell lines. A. CatK expression in human PCa tissues. Immunohistochemical staining was performed for detection of CatK in human PCa and corresponding non-neoplastic tissues. **B.** Total RNA was extracted from prostate epithelial cells (PrEC), LNCaP, C4-2B, and PC3 cells using TRIzol reagent (Life Technologies), then subjected to RT-PCR for detection CatK mRNA. PCR product of 399 bp is detected. **C.** Quantification of CatK mRNA determined by real-time PCR. Internal control is β -actin. **D.** To evaluate for CatK expression in the prostate cancer cells, confluent PrEC, LNCaP, C4-2B, and PC3 cells were washed with PBS and then lysed in RIPA buffer. Proteins (40μg/lane) were applied to SDS-PAGE followed by Western blot system with rabbit anti-human CatK polyclonal Ab (Santa Cruz), or mouse anti-human β-actin monoclonal Ab (Sigma). The Ab binding was revealed using an HRP-conjugated anti-rabbit IgG, or HRP-conjugated anti-mouse IgG1 (Santa Cruz) and enhanced chemiluminescence (ECL) blot detection system (Amersham Pharmacia Biotech, Poscataway, New Jersey). **E.** To test CatK production in the CaP cell supernatants, CM collected from PrEC, LNCaP, C4-2B, and PC3 cell cultures were measured by an ELISA kit (Alpco, Windham, NH). PrEC cells are prostate epithelial cell contain normal human epithelial cells purchased from Cambrex BioSciences.

Then, we observed the inhibitory effect of CatK inhibitor on the PCa-induced bone resorption in vitro (Figure 2).

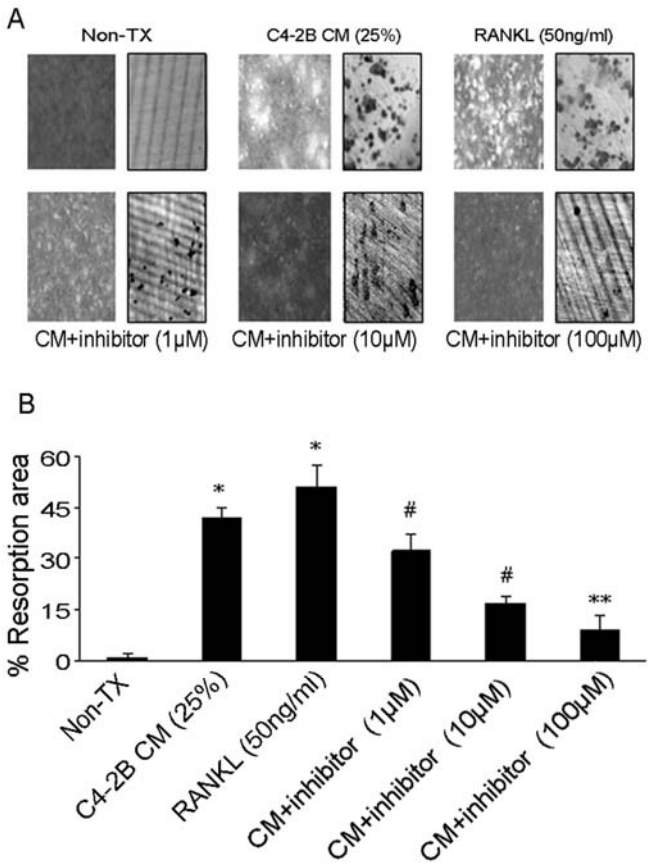

Figure 2. CatK inhibitor diminishes PCa-CM-induced bone resorption in vitro . To evaluate the ability of the CatK inhibitor to diminish the CaP-CM-induced bone resorption, we first collected CM from C4-2B cell culture. Then we seeded murine osteoclast precusor RAW 264.7 cells into the 24-wells of BD Biocate osteologic bone cell culture system that consist of sub-micro synthetic calcium phosphate thin films coated onto the culture vessels or 96-well plate with dentin slices. The soluble RANKL (50ng/ml) or the indicated concentration of CatK inhibitor was added. The dentin slices were stained with TRAP staining kit and then the cells on the dentin slices were then removed and area of resorption was determined microscopically with SPOT software. **A.** Representative images of resorption pits on dentin slices or synthetic calcium phosphate thin films are shown. **B.** Samples were evaluated in triplicates. Results are reported as mean (±SD). *P<0.001 compared to Non-TX group; #P<0.01 C4-2B CM-treated group; **P<0.001 compared to C4-2B CM-treated group. We observed that C4-2B CM induced bone resorption and this induction was diminished by CatK inhibitor in a dose-dependent manner.

Next, we observed the inhibitory effect of CatK inhibitor on the PCa cell invasion suggesting the role of CaK in PCa tumor invasion (Figure 3).

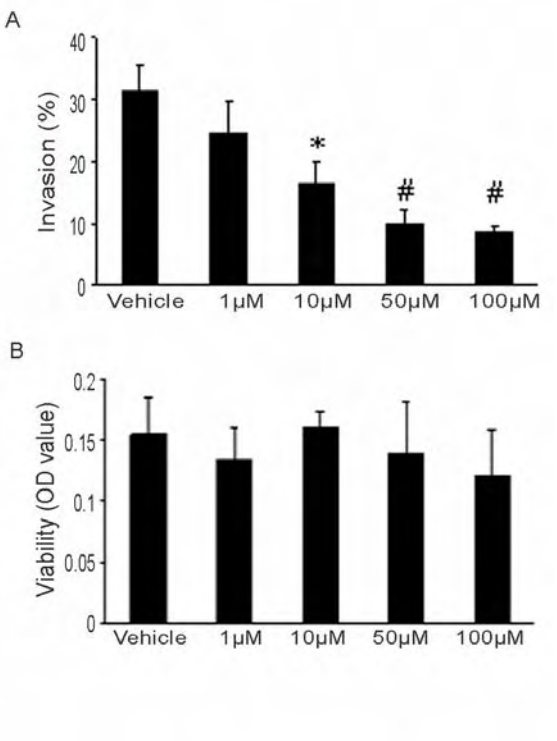

Figure 3. CatK inhibitor diminishes the invasiveness of C4-2B cells. A. The *in vitro* invasion assay was performed using C4-2B cells cultured in 24-well transwell chambers (BD BioCoat Matrigel Invision Chamber, BD Biosciences, MA), as directed by the manufacturer. Cell penetration (after 24 hours of culture with vehicle or different concentration of the inhibitor) through the membrane was detected by staining the cells on the porous membrane with a Diff-Quik stain kit and quantified by counting the numbers of cells that penetrated the membrane in five microscopic fields (at x200 magnification) per filter. Invasive ability was defined as the proportion of cells that penetrated the matrix-coated membrane divided by the number of cells that migrated through the uncoated membrane (baseline migration). The results are reported as the mean of triplicate assays. **B.** The C4-2B cells viability were examined by MTS assay. C4-2B cells were treated with various doses of CatK inhibitor (the doses that was used in the tumor invision assay). We did not observe any significant differences among these cells in terms of cell viability.

Finally, we injected C4-2B cells into the tibiae of SCID mice and then the animals received either vehicle or Cat K inhibitor for 8 weeks either at the time of tumor cells injection (tumor establishment model) or 4 weeks after tumor cells were injected (tumor progression model). In the tumor establishment model, CatK inhibitor significantly prevented the establishment of mixed osteolytic/osteoblastic tibial tumors as were observed in vehicle-treated animals. In the progression model, CatK inhibitor diminished tumor-induced bone lesions.

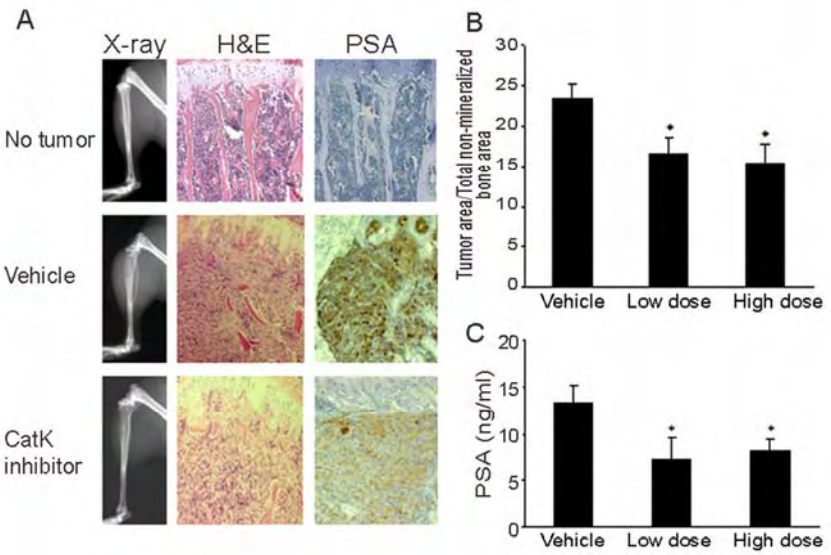

Figure 4. CatK inhibitor prevents establishment of PCa tumor in bone. C4-2B cells were directly injected into the tibia of SCID mice (n=10/per group). At the time of tumor injection, either CatK inhibitor (100 or 50mg/kg/day) or vehicle was orally administrated daily for 12-weeks. Mice were sacrificed at the end of 12 weeks. X-ray, H&E, and PSA were determined. **A.** In this representive figure, note the area of osteolysis and osteosclerosis of the vehicle treated mouse compared to the radiograph of the CatK inhibitor treated mouse. PSA is strong positive in all vehicle treated mice compared to CatK inhibitor treated mice. **B.** Bone histomorphometry results. *P<0.01 compared to vehicle group. **C.** Serum PSA levels in the mice model were measured by ELISA and found CatK inhibitor decreased serum levels. (For the tumor progression model, we observed the similar results)

In our next step, we will add groups of animals that will use bisphosphonates Zometa alone or combine the oral CatK inhibitor with Zometa. The rationale for this is based on limitation of using bisphosphonates in the treatment of skeletal metastases. By adding other anti-resorptive therapies, such as a cathepsin-K inhibitor that functions through a different mechanism of action than bisphosphonates, may block bone resorption and thus further improve the outcome of CaP skeletal metastases and reduce the adverse effects.

Key Research Accomplishments

- Confirmation of CatK expression in PCa LNCaP, C4-2B, and PC3 PCa cell lines (as well as in tissues)
- We found inhibitory effect of CatK inhibitor on the PCa cell invasion
- In the tumor establishment model, CatK inhibitor significantly prevented the establishment of mixed osteolytic/osteoblastic tibial tumors as were observed in vehicle-treated animals.
- In the progression model, CatK inhibitor diminished tumor-induced bone lesions.
- These above findings set up a strong background for our further studies that is using CatK inhibitor in combination with ZA, to investigate the potential synergistic effects.

Reportable Outcomes

This work has been presented during the Annual meeting of American Association for Cancer Research in San Diego, CA April 2008 (see appendix).

The manuscript is in preparation and will be submitted within 3-6 months.

Conclusion

We conclude that CatK inhibitor is an effective drug to prevent the establishment and diminish progression of PCa growth in bone.

References

Yi Lu, Evan T. Keller, Qiuyan Chen, Jinlu Dai, June Escara-Wilke, Eva Corey, Johann Zimmermann, Jian Zhang. Targeting cathepsin K diminishes prostate cancer cell establishment and growth in murine bone. 2008, AACR Proceedings, 49: 872

Appendices

Please see the attached copy of AACR poster.

Supporting data

N/A

Targeting cathepsin K diminishes prostate cancer cell establishment and growth in murine bone

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[1]Department of Medicine, University of Pittsburgh, Pittsburgh, PA 15232; [2]Department of Urology and Pathology, University of Michigan, Ann Arbor, MI 48109; [3]Department of Nasopharyngeal Carcinoma, Cancer Center, Sun Yat-sen University, Guangzhou, 510060, China; [4]Department of Urology, University of Washington, Seattle, Washington 98195; [5]Novartis Pharma Ltd., Basel, Switzerland

Abstract

The prostate cancer (PCa) invasion and metastasis processes are facilitated by proteolytic cascade involving multiple proteases, including matrix metalloproteinases (MMPs), serine proteases (uPA, plasmin), and cysteine proteases such as cathepsin K (CatK). CatK degrades collagen I and expresses predominantly in osteoclasts. Recently CatK expression has been shown in breast and prostate cancer cells. Importantly, its expression level is higher in PCa bone metastatic sites than primary tumor or normal prostate. The role of CatK in PCa skeletal metastasis, however, has not been studied. In this study, we first confirmed the expression of CatK in LNCaP, C4-2B, and PC3 PCa cell lines. Then, we observed the inhibitory effect of CatK inhibitor on the PCa cell invasion suggesting the role of CaK in PCa tumor invasion. Finally, we injected C4-2B cells into the tibiae of SCID mice and then the animals received either vehicle or Cat K inhibitor for 8 weeks either at the time of tumor cells injection (tumor establishment model) or 4 weeks after tumor cells were injected (tumor progression model). In the tumor establishment model, CatK inhibitor significantly prevented the establishment of mixed osteolytic/osteoblastic tibial tumors as were observed in vehicle-treated animals. In the progression model, CatK inhibitor diminished tumor-induced bone lesions, as demonstrated by radiograph, histology and bone histomorphometry. CatK inhibitor also decreased serum PSA levels in both animal models indicating the decreased tumor burden. We conclude that CatK inhibitor is an effective drug to prevent the establishment and diminish progression of PCa growth in bone.

Background

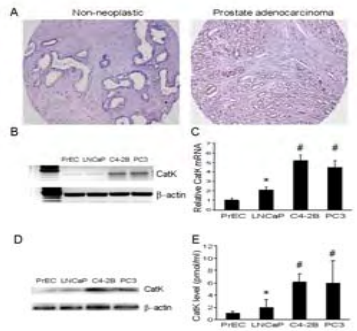
•Clinical PCa frequently metastasizes to bone resulting in predominantly osteoblastic lesions, but with underlying osteoclast-mediated bone resorption.
•Cathepsin K (CatK) is a cysteine protease that plays an essential role in osteoclast function and in the degradation of protein components of the bone matrix by cleaving proteins such as collagen type I, collagen type II and osteonectin. Because of its ability to destroy matrix components, CatK and some of its family members have been implicated in diseases involving bone and cartilage destruction, including tumor invasion and rheumatoid arthritis.
•Recently, CatK was found in breast cancer cells that are capable of bone resorption. The CatK mRNA was also detected in PCa cell lines and in primary PCa and metastases.
•Selective human CatK inhibitors have been described that potently inhibiting osteoclast resorption both in vitro and in vivo.
•Zometa has significant efficacy in the treatment of bone metastases, However it has limitations (J Natl Cancer Inst, *96:* 879-882, 2004. J Natl Cancer Inst, *94:* 1458-1468, 2002)
•Therefore, new therapeutic medications are urgently needed for PCa skeletal metastasis.

Hypothesis

•CatK contributes to PCa-induced osteoclast activity at bone metastatic sites.
•Inhibition of CatK by its selective inhibitor may prevent the PCa establishment and progression in bone.

Results

Cathepsin K express in the prostate cancer tissues and cell lines



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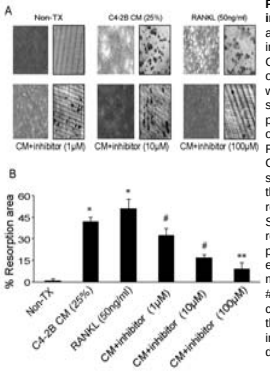
Cathepsin K specific inhibitor diminishes PCa cell-induced bone resorption



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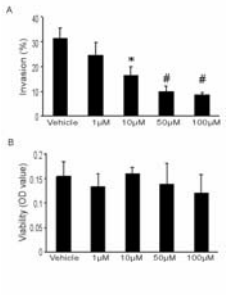
Cathepsin K specific inhibitor diminishes PCa cell invasion



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Cathepsin K inhibitor prevents PCa tumor establishement *in vivo*

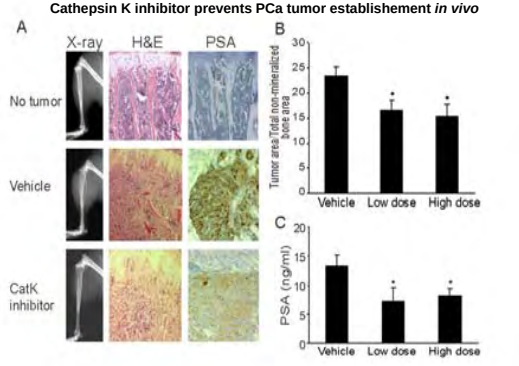

Figure 4. CatK inhibitor prevents establishment of PCa tumor in mouse bone. C4-2B cells were directly injected into the tibia of SCID mice (n=10/per group). At the time of tumor injection, either CatK inhibitor (100 or 50mg/kg/day) or vehicle was orally administrated daily for 12-weeks. Mice were sacrificed at the end of 12 weeks. X-ray, H&E, and PSA were determined. **A.** In this representive figure, note the area of osteolysis and osteosclerosis of the vehicle treated mouse compared to the radiograph of the CatK inhibitor treated mouse. PSA is strong positive in all vehicle treated mice compared to CatK inhibitor treated mice. **B.** Bone histomorphometry results. *P<0.01 compared to vehicle group. **C.**Serum PSA levels in the mice model were measured by ELISA and found CatK inhibitor decreased serum levels. (For the tumor progression model, we observed the similar results, not shown)

Conclusion

•Using CatK inhibitor is an effective approach to prevent and diminish progression of PCa growth in bone.
•This novel targeting strategy has provided rationale to inhibit PCa-induced bone resorption in clinical trails.

Acknowledgments

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